Final Scientific Report

Cover Page

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TITLE: Host ammonification by postharvest pathogens and its contribution to fungal colonization and symptom development

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Keywords: **Colletotrichum, alkalinization, pathogenicity**

Abbreviations commonly used in the report, in alphabetical order**:**

Budget: IS: $227,000 US: $103,000 Total: $330,000

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Principal Investigator

Signature
Authorizing Official, Principal Institution

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged	4	1	6	11
Submitted, in review, in preparation	1			1
Invited review papers				
Book chapters			1	1
Books			1	1
Master theses				
Ph.D. theses		1	2	3
Abstracts			4	4
Not refereed (proceedings, reports, etc.)		1		1

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Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings		1	4	4
Longer Visits (Sabbaticals)				

Description Cooperation:
Cooperation between the American and Israeli team was very high. Several aspect of the work was done in joint collaborations specially since one student of the American scientist follow several methods similar to the those developed by the Israeli partner. But we had independent research in each specific pathogen. Beside that very fruitful work was done and published together. We intend to follow this work and present a future joint proposal.

Abstract

Postharvest decay of fruits and vegetables caused by pathogenic and saprophytic fungi significantly impairs the quality and quantity of fresh produce brought to market. Consequently, there is considerable interest in identifying factors that determine the susceptibility of these commodities to pathogen infection. Insidious postharvest decays remain quiescent during fruit growth and harvest, but activate during the postharvest period. A key response to the physiological changes occurring during fruit ripening is the initiation of ammonium secretion by the pathogen. Ammonium ions at the infection site (ammonification) have subsequent effects on both the pathogen and the host. An accompanying alkalinization process resulting from ammonia accumulation contributes to pathogenicity, since some important fungal virulence factors, (such as pectate lyase in *Colletotrichum sp.*), are significantly expressed only under alkaline conditions. In this proposal, investigated the mechanisms by which ammonification and alkalinization of infected tissues by the pathogen affect the host's defense response to fungal attack, and instead increase compatibility during postharvest pathogen-host interactions. **Our hypotheses were: 1)** that host signals, including ripening-related changes, induce secretion of ammonia by the pathogen; **2)** that ammonia accumulation, and the resultant environmental alkalinization regulate the expression of fungal virulence genes that are essential for postharvest rot development; **3)** that ammonification enhanced fungal colonization, by "suppression of host responses", including production of reactive oxygen species, activation of superoxide, and polyphenol oxidase production.

Our objectives were: to analyze: **1)** factor(s) which activate the production and secretion of ammonia by the fungus; **2)** fungal gene(s) that play role(s) in the ammonification process; **3)** the relationship between ammonification and the activation of host defense response(s) during pathogen colonization; and **4)** analyze host gene expression in alkalinized regions of fruits attacked by hemibiotrophic fungi.

The specific objectives of the proposal were to analyze:

1) Factor which activate the production and secretion of ammonia by the fungus.

2) Fungal gene(s) that play role(s) in the ammonification process.

3) The relationship between ammonification and the activation of host defense response(s) during pathogen colonization.

4) Analyze host gene expression in alkalinized regions of fruits attacked by hemibiotrophic fungi.

Major conclusions

1. We determined that the initial acidic pH of the fruit is conducive to ammonia secretion and the subsequent alkalinization of the infection site, and facilitates fungal virulence and the transformation from the quiescent-biotrophic to active-necrotrophic state.

2. Host-tissue alkalinization via ammonia accumulation is key to *Colletotrichum* spp. colonization. Using macroarrays carrying *C. gloeosporioides* cDNAs, we identified a set of genes involved in synthesis and catabolism of ammonia accumulation during the alkalinization process.

3. Our results show that *C. coccodes* activates host reactive oxygen species and H_2O_2 production through ammonium secretion. The resultant enhancement in host tissue decay is an important step in the activation of the necrotrophic process needed for colonization.

4. Common-up-regulated genes showed over representation of pathogen related (PR) proteins, salicylic acid (SA) dependent and systemic acquired resistance (SAR) related genes as well as genes related to biotic stress. The down regulated genes showed over representation of Jasmonic acid (JA) dependent genes. Indeed, direct application of SA to the fruit enhanced *C. coccodes* necrotrophic colonization, whereas the application of JA delayed colonization. Importantly, green resistant fruit and susceptible red fruit displayed similar gene expression patterns. This suggests that quiescence and active colonization by the fungus induce the same fruit response pathways. The results imply that, in a manner independent of fruit maturity, ammonia accumulation activates SA and suppress JA pathways through activation of host RBOH and in green resistant fruit additional factors probably contribute to inhibition of fungal growth.

Agricultural implications

In work that has been done in a parallel systems it was described a clear implication of the pH modulation in pathogenicity of *Colletotrichum gloeosporioides* and its involvement in colonization of postharvest diseases. These results further support the early reports in the former BARD supporter project, that indicate that the alkalinization process is a significant factor for *C. gloeosporioides* pathogenesis and that treatment with acid solutions that modulate host environment are very efficient for the prevention of decay development.

Achievements

Recent publications have suggested that environmental pH is considered a key factor in determining pathogen compatibility. Our early hypothesis was that the environmental pH at the infection site, which is dynamically controlled by activities of both the host and the pathogen, regulates the expression of genes necessary for disease development in *Colletotrichum sp.* This form of regulation ensures that genes are expressed at optimal conditions for their encoded activities. Pectate lyase encoded by *pelB*, has been demonstrated to play a key role in virulence of *C. gloeosporioides* in avocado fruit. In this proposal we have examined, 1) the mechanisms employed by these fungi to establish a suitable pH environment, 2) the molecular levels at which genes and gene products are regulated in response to environmental pH, and 3) the molecular basis and functional importance of pH-responsive gene regulation during pathogenicity.

Our first objective was to determine the factor which activate the production and secretion of ammonia by the fungus. *Colletotrichum coccodes* was found to alkalinize the decaying tissue of tomato fruits via accumulation and secretion of ammonia. Alkalinization dynamics caused by ammonia secretion from growing hyphae was examined microscopically using the pH-sensitive fluorescent dye BCECF (2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein). Values of pH of 7.9 observed in the host tissue close to the hyphal tips, declined to pH 6.0, at 10 mm away from the hyphal tip, which was a value that was still higher than that detected in the healthy tissue, pH 4.2. Ammonia accumulation at the infection site depended on the initial environmental pH. Treatments with low (4.0) pH buffer at the infection site resulted in high levels of ammonia secretion and increased virulence of *C. coccodes* compared a similar treatments with buffer at pH 7.0. Significantly, mutants of *C. coccodes* defective in nitrogen utilization, *nit*$^{-}$ and *areA*$^{-}$, are impaired in ammonia secretion and showed reduced decay development. The reduced infection rate of *nit*$^{-}$ mutants could be complemented by adding glutamine at the infection site. Thus, ammonia accumulation is a critical factor contributing to *C. coccodes* pathogenicity on tomato fruits. The results show that the initial acidic pH of the fruit is conducive to ammonia secretion and the subsequent alkalinization of the infection site, and facilitates fungal virulence and the transformation from the quiescent-biotrophic to active-necrotrophic state.

Our second objective was to determine the fungal gene(s) that play role(s) in the ammonification process. Host-tissue alkalinization via ammonia accumulation is key to *Colletotrichum* spp. colonization. Using macroarrays carrying *C. gloeosporioides* cDNAs, we monitored gene expression during alkalinization process. A set of genes involved in synthesis and catabolism of ammonia accumulation were identified.

Our third objective was to determine the relationship between ammonification and the activation of host defense response(s) during pathogen colonization.

Colletotrichum pathogens of fruits and leaves are known ammonium secretors. Here we show that *C. coccodes* virulence, as measured by tomato (*Solanum lycopersicum* cv. Motelle) fruit tissue necrosis, correlates with the amount of ammonium secreted. Ammonium application to fruit tissue induced hydrogen peroxide accumulation. To examine whether the tomato NADPH oxidase, SlRBOH, is a source for the ammonium-induced H_2O_2, wild-type and antisense lines abrogated for SlRBOH (SlRBOH-AS) were examined. Wild-type lines produced 7.5-fold more reactive oxygen species when exposed to exogenous ammonium than did SlRBOH-AS lines. *C. coccodes* colonization of wild-type tomato lines resulted in higher H_2O_2 production and faster fungal growth rate compared to colonization in the SlRBOH-AS mutant, although the amount of ammonium secreted by the fungi was similar in both cases. Enhanced ion leakage and cell death of fruit tissue were correlated with H_2O_2 accumulation, and treatment with the reactive oxygen scavenger N-acetyl-L-cysteine decreased H_2O_2 production, ion leakage and cell death. Importantly, the activation of reactive oxygen species production by ammonium was positively affected by an extracellular pH increase from 4 to 9, implying that ammonium exerts its control via membrane penetration. Our results show that *C. coccodes* activates host reactive oxygen species and H_2O_2 production through ammonium secretion. The resultant enhancement in host tissue decay is an important step in the activation of the necrotrophic process needed for colonization.

Our fourth objective was to analyze host gene expression in alkalinized regions of fruits attacked by hemibiotrophic fungi. Insidious fungal infections by postharvest pathogens such as *Colletotrichum* remain quiescent after infection of unripe-green fruit. However, during ripening and senescence, the pathogens adopt a necrotrophic life style, rapidly colonizing the tissue. *Colletotrichum coccodes* secretes ammonium during germination and colonization of host tissue. Here we show that ammonium accumulation induced integrity changes in the host

nucleus and programmed cell death (PCD). To establish the direct role of ammonia in this process, transcriptomes of fruit tissue treated with ammonia and of tissue from the leading edge of fungal colonization was compared. The analysis revealed 82 and 237 common up-regulated and common down-regulated genes, respectively. RT-PCR analysis of select transcripts in normal and transgenic RBOH antisense plants revealed that their expression was RBOH-dependent. Common-up-regulated genes showed over representation of pathogen related (PR) proteins, salicylic acid (SA) dependent and systemic acquired resistance (SAR) related genes as well as genes related to biotic stress. The down regulated genes showed over representation of Jasmonic acid (JA) dependent genes. Indeed, direct application of SA to the fruit enhanced *C. coccodes* necrotrophic colonization, whereas the application of JA delayed colonization. Importantly, green resistant fruit and susceptible red fruit displayed similar gene expression patterns. This suggests that quiescence and active colonization by the fungus induce the same fruit response pathways. The results imply that, in a manner independent of fruit maturity, ammonia accumulation activates SA and suppress JA pathways through activation of host RBOH and in green resistant fruit additional factors probably contribute to inhibition of fungal growth.

Overall this project has contributed to our understanding of the role of ambient pH and ambient pH-dependent gene expression on pathogenic development in Colletotrichum. Other closely related research was carried out as well in relation to another Colletotrichum species, *C. graminicola* and in this system, while pH changes were induced by the fungus, the changes did not correlate with the ammonia accumulation. Furthermore nit mutants did not show and differential pathogenicity compared to the WT, as was found in *C. gloeosporioides* (Alkan et al., 2008), suggesting some likely different behavior between the *C. gloeosporioides* and *graminicola* that should be addressed in the future.

Concerning the cooperation this proposal has been a clear basis for joint cooperation. Several parts of the work on *C. graminicola* in the US and *C. gloeosporioides* in Israel were carried out in parallel in both labs by the joint discussions of Mrs Maria F. Torres, a student of Dr. Lisa Vaillancourt and the students in Israel, Dr. Noam Alkan. Scientific meetings took place during several of the annual Phytopathological and Fungal Genetics meetings beside of a specific visit of D. Prusky to Kentucky.

Appendix

Table of contents:

Published papers.

1. Alkan, N., Davydov, O., Sagi,M., Fluhr. R., and Prusky, D. 2009. Ammonium secretion by *Colletotrichum coccodes* activates host NADPH oxidase activity enhancing host cell death and fungal virulence in tomato fruits. Molecular Plant Microbe Interactions 22: 1484–1491
2. Davidzon M., Kobiler, I., Alkan N., and Prusky D. 2009 Acidification of fruit environment by gluconic acid during decay development of *Phomopsis mangiferae*. Postharvest Biology Technology 55: 71–77.
3. Prusky D., M. Lodovica Gullino. 2009. Plant Pathology in the 21st Century: Contributions to the 9th International Congress. Volume 2: Post-Harvest Pathology. Springer Verlag.
4. Prusky,D., Alkan, N., Miyara, I., Barad, S., Davidzon, M., Kobiler, I., Brown-Horowitz, S., Lichter, A., Sherman, A., and Fluhr, R. 2009. Mechanisms Modulating Postharvest Pathogen Colonization of Decaying Fruits. In Plant Pathology in the 21st Century. Editor Prusky D., M. Lodovica Gullino. Springer Verlag**.** 43-54
5. Miyara, I., Davidzon, M., Sherman, A., and Prusky, D. 2010. pH Regulation of ammonia secretion by Colletotrichum gloeosporioides and its effect on appressorium formation and pathogenicity. Molecular Plant Microbe Interactions 23:304-316.
6. Kobiler,I., Akerman,M., Huberman, L., and Prusky, D. 2010. Integration of pre- and postharvest treatments for the control of black spot caused by *Alternaria alternata* in stored persimmon fruits. Postharvest Biology and Technology 59: 166–171
7. Alkan, N., Fluhr, R., and Prusky, D. 2010. *Colletotrichum coccodes* infection of ripe and unripe tomato fruit is modulated by ammonium secretion through salicylic and jasmonic acid pathways leading to PCD and differential colonization. Submitted to MPMI